Altered activation of endothelial anti- and proapoptotic pathways by high-density lipoprotein from patients with coronary artery disease: role of high-density lipoprotein-proteome remodeling

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Abstract: BACKGROUND: Endothelial dysfunction and injury are thought to play an important role in progression of coronary-artery-disease (CAD). High-density-lipoprotein from healthy subjects (HDL-Healthy) has been proposed to exert endothelial anti-apoptotic effects that may represent an important anti-atherogenic property of the lipoprotein. The present study therefore aimed to compare effects of HDLCAD and HDLHealthy on activation of endothelial anti- and pro-apoptotic pathways and to determine which changes of the lipoprotein are relevant for these processes. METHODS AND RESULTS: HDL was isolated from patients with stable CAD (HDLsCAD), an acute coronary syndrome (HDLACS) and healthy subjects. HDLHealthy induced expression of the endothelial anti-apoptotic Bcl-2 protein Bcl-xL and reduced endothelial cell apoptosis in vitro and in apoE-deficient-mice in vivo. In contrast, HDLsCAD and HDLACS did not inhibit endothelial apoptosis, failed to activate endothelial Bcl-xL and stimulated endothelial pro-apoptotic pathways, in particular p38-MAPK-mediated activation of the pro-apoptotic Bcl-2-protein tBid. Endothelial anti-apoptotic effects of HDLHealthy were observed after inhibition of endothelial nitric-oxide-synthase and after delipidation, but not completely mimicked by apoA-I or reconstituted HDL, suggesting an important role of the HDL-proteome. HDL proteomics analyses and subsequent validations and functional characterizations suggested a reduced clusterin- and increased apoC-III-content of HDLsCAD and HDLACS as mechanisms leading to altered effects on endothelial apoptosis. CONCLUSIONS: The present study demonstrates for the first time that HDLCAD does not activate endothelial anti-apoptotic pathways, but rather stimulates potential endothelial pro-apoptotic pathways. HDL-proteome remodeling plays an important role for these altered functional properties of HDL. These findings provide novel insights into mechanisms leading to altered vascular effects of HDL in coronary disease.

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Altered activation of endothelial anti- and pro-apoptotic pathways by high-density lipoprotein from patients with coronary artery disease: Role of HDL-proteome remodeling

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ABSTRACT

Background: Endothelial dysfunction and injury are thought to play an important role in progression of coronary-artery-disease (CAD). High-density-lipoprotein from healthy subjects ($HDL_{Healthy}$) has been proposed to exert endothelial anti-apoptotic effects that may represent an important anti-atherogenic property of the lipoprotein. The present study therefore aimed to compare effects of HDL_{CAD} and $HDL_{Healthy}$ on activation of endothelial anti- and pro-apoptotic pathways and to determine which changes of the lipoprotein are relevant for these processes.

Methods and Results: HDL was isolated from patients with stable CAD (HDL_{sCAD}), an acute coronary syndrome (HDL_{ACS}) and healthy subjects. $HDL_{Healthy}$ induced expression of the endothelial anti-apoptotic Bcl-2 protein Bcl-xL and reduced endothelial cell apoptosis *in vitro* and in apoE-deficient-mice *in vivo*. In contrast, HDL_{sCAD} and HDL_{ACS} did not inhibit endothelial apoptosis, failed to activate endothelial Bcl-xL and stimulated endothelial pro-apoptotic pathways, in particular p38-MAPK-mediated activation of the pro-apoptotic Bcl-2-protein tBid. Endothelial anti-apoptotic effects of $HDL_{Healthy}$ were observed after inhibition of endothelial nitric-oxide-synthase and after delipidation, but not completely mimicked by apoA-I or reconstituted HDL, suggesting an important role of the HDL-proteome. HDL proteomics analyses and subsequent validations and functional characterizations suggested a reduced clusterin- and increased apoC-III-content of HDL_{sCAD} and HDL_{ACS} as mechanisms leading to altered effects on endothelial apoptosis.

Conclusion: The present study demonstrates for the first time that HDL_{CAD} does not activate endothelial anti-apoptotic pathways, but rather stimulates potential endothelial pro-apoptotic pathways. HDL-proteome remodeling plays an important role for these altered functional properties of HDL. These findings provide novel insights into mechanisms leading to altered vascular effects of HDL in coronary disease.

Key Words: High-density lipoprotein, apoptosis, proteomics, clusterin, apolipoprotein C-III

INTRODUCTION

Reduced plasma levels of HDL cholesterol are associated with an increased risk of coronary artery disease (CAD).[1] Moreover, in patients with CAD that are treated with statin and have low levels of low-density lipoprotein (LDL) cholesterol, reduced HDL cholesterol levels were predictive of major cardiovascular events.[2] Besides promoting reverse cholesterol transport,[3, 4] HDL has been demonstrated to exert anti-atherosclerotic effects, including anti-inflammatory properties and stimulation of endothelial nitric oxide (NO) production.[5-9] However, these effects of HDL have been observed to be highly heterogenous in patients with CAD or diabetes.[10-12]

Endothelial dysfunction and injury are thought to contribute importantly to the progression of CAD.[13-15] Experimental studies have indicated that atherosclerotic lesion-prone vascular regions are characterized by a high endothelial cell turn-over,[16] which has been attributed to an increased rate of endothelial cell apoptosis. Moreover, superficial atherosclerotic plaque erosion with loss of an intact endothelial cell monolayer is observed quite frequently in patients with an acute coronary syndrome (ACS) based on pathological[17, 18] as well as on observations from clinical high-resolution intracoronary imaging studies.[19] In pathological studies of coronary atherosclerotic plaque erosion thrombi were observed in direct contact with the intima in areas with absent endothelium likely promoting disease progression.[17] Endothelial cell apoptosis has therefore been suggested to contribute importantly to the pathophysiology of coronary disease.[20-22] The capacity of HDL to attenuate endothelial cell apoptosis may therefore represent a potentially important anti-atherogenic property of HDL.[23-26]

However, as described above, the vascular effects of HDL can be highly variable in patients with cardiovascular disease, a phenomenon referred to as "HDL dysfunction".[10, 12, 27] The present

study was therefore designed to compare the effects of HDL from patients with stable CAD or an ACS and healthy subjects on endothelial anti-apoptotic and pro-apoptotic signaling pathways. Moreover, we aimed to characterize alterations in HDL from patients with CAD that may lead to altered functional properties of HDL with respect to activation of endothelial anti-apoptotic and pro-apoptotic signaling pathways.

METHODS

An expanded description of the methods is available in Supplementary Methods.

Patient Characteristics. Patients with stable CAD or an ACS (STEMI or NSTEMI) and healthy subjects (without cardiovascular risk factors) were recruited at the University Hospital of Zurich. Exclusion criteria were accompanying infectious, inflammatory or autoimmune disorders, advanced kidney or liver failure, diabetes, neoplastic disorders and a history of major surgery or trauma within the previous month.

Isolation of High-Density Lipoprotein and HDL-delipidation. HDL was isolated by either sequential ultracentrifugation (d=1.063-1.21g/ml) or gel filtration chromatography. For proteomics studies and functional studies HDL was delipidated using methanol/chloroform extraction. Detailed description of the methods is provided in Supplementary Methods.

Measurement of Endothelial Cell Apoptosis *In Vitro*. Endothelial apoptosis was measured by FACS analysis with annexin-V-staining or TUNEL assay, as well as using fluorescence microscopy with annexin-V-staining. Caspase-3 activity was measured using a colorimetric assay. Detailed description of the methods is provided in Supplementary Methods.

Measurement of Endothelial Cell Apoptosis *In Vivo* using FACS Analysis and Active Caspase-3 Staining. Male apoE(-/-).C57BL/6 mice, aged 12-16 weeks, were used for tail-vein injection of HDL (14mg HDL-protein/kg body-weight), following anaesthesia with inhalation of isoflurane(3%). Twenty-four hours after the injection of HDL or PBS-buffer, mice were

euthanized and the aorta were harvested and immediately digested for FACS analysis of endothelial cell apoptosis or fixated for histological staining. For detailed description of the methods, please refer to the Supplementary Methods.

Biochemical Validation of HDL-associated Proteins. ELISA was used for validation of changes in clusterin-(BioVendor R&D, USA) and apoC-III-(AssayPro, USA) content of HDL.

Western Blot analysis. Phosphorylation of Akt at serine residue473, phosphorylation of MAPK-p38 and expression of Bcl-2, Bcl-xL, tBid, Bak were determined by western blot analysis. Anti-human phospho-Akt(Ser473), anti-human total Akt, anti-human phospho-p38-MAPK, anti-human total p38-MAPK were purchased from Cell Signaling Technology. Antibodies against human Bcl2, Bcl-xL, tBid and Bak were from Cell Signaling Technology.

Statistical Analysis. All data are expressed as mean±SEM. All analyses were performed with SPSS 21.0 (IBM SPSS). Significance was tested using Student's-*t*-test and ANOVA with Dunnett *post-hoc* test for multiple comparison analysis. A value of $p<0.05$ was considered statistically significant.

RESULTS

HDL was isolated from patients with coronary disease (HDL_{CAD}), with either stable CAD (HDL_{sCAD}) or an acute coronary syndrome (HDL_{ACS}), and age- and gender-matched healthy subjects ($HDL_{Healthy}$). The characteristics of the study population are shown in Table 1.

Effects of $HDL_{Healthy}$ and HDL_{CAD} on endothelial cell apoptosis *in vitro* and *in vivo*

$HDL_{Healthy}$ markedly reduced endothelial cell apoptosis *in vitro*, whereas in marked contrast, HDL_{sCAD} or HDL_{ACS} did not limit endothelial apoptosis induced by serum and growth factor deprivation (Figures 1A-C,E-F). Endothelial caspase-3 activity was reduced by $HDL_{Healthy}$, but not after treatment with HDL_{sCAD} or HDL_{ACS} (Figure 1D). Similarly, detection of fragmented DNA using TUNEL staining showed significantly attenuated endothelial apoptosis by $HDL_{Healthy}$ (26.7%±12.0% vs control 41.6%±10.3%, $p<0.05$), but not HDL_{sCAD} or HDL_{ACS} (36.9%±9.1% and 32.2%±13.6%, respectively). Furthermore, $HDL_{Healthy}$ but not HDL_{sCAD} or HDL_{ACS} reduced endothelial apoptosis induced by another stimulus, i.e. TNF-α, as measured by annexin-V staining (Figure 1G) or TUNEL assay (apoptotic endothelial cells after $HDL_{Healthy}$ vs. HDL_{CAD} 23.02%±14.4% vs. 39.7%±13.7%; $p<0.05$). The rate of TNF-α-induced endothelial apoptosis was similar as compared to previous studies, such as the study by Spyridopoulos et al.[28] reporting 39.3% apoptotic endothelial cells after TNF-α exposure.

Moreover, we examined the capacity of HDL to impact on endothelial apoptosis *in vivo* by injecting isolated HDL to apoE-/- mice. Administration of $HDL_{Healthy}$ (14mg HDL-protein/kg body weight) to apoE-/- mice reduced endothelial apoptosis in the aorta (harvested after 24hours) while HDL_{CAD} did not reduce endothelial apoptosis as determined by Annexin-V measurement

as well as immunofluorescence staining with TUNEL and active caspase-3 assay (Figure 2A-E). In these studies, we did not observe a significant difference between the anti-apoptotic capacity of HDL_{sCAD} as compared to HDL_{ACS} (7.2±3.1% vs 6.3±3.8%; p=0.51).

$HDL_{Healthy}$ reduces endothelial apoptosis after inhibition of endothelial NO-synthase

Endothelial NO synthase is a potentially important regulator of endothelial apoptosis and effects of HDL on eNOS are adversely affected in patients with CAD.[10, 29] We therefore examined whether the effects of HDL on endothelial cell apoptosis are critically dependent on eNOS. Notably, inhibition of eNOS-mediated NO production by both, pharmacological inhibition with N-nitro-L-arginine methyl ester (L-NAME) and eNOS-specific RNA interference did not prevent endothelial anti-apoptotic effects of $HDL_{Healthy}$ (Figure 3A), suggesting that HDL activates endothelial anti-apoptotic pathways independent of eNOS.

Role of protein moiety for endothelial anti-apoptotic capacity of $HDL_{Healthy}$

To investigate which alterations of the HDL particle may impair the capacity to limit endothelial apoptosis, we examined effects of the HDL protein fraction (delipidated HDL), rHDL, purified apoA-I and recombinant apoA-I on endothelial apoptosis. Interestingly, delipidated $HDL_{Healthy}$ exerted a potent endothelial anti-apoptotic activity as measured by annexin-V staining (Figure 3B). The endothelial anti-apoptotic effects of delipidated $HDL_{Healthy}$ were more profound as compared to purified or recombinant apoA-I or rHDL (Figure 3B), suggesting an important role of the HDL proteome and its remodeling for altered effects of HDL_{CAD} on endothelial apoptosis. Similarly, by using TUNEL staining we observed that $HDL_{Healthy}$ and delipidated $HDL_{Healthy}$ significantly reduced the number of apoptotic endothelial cells (22.2%±8.0% and 19.0%±14.1%

vs control 37.6±10,2%, p<0.05), that was not observed with apoA-1 or rHDL alone (29.0%±8.7% and 25.3%±10.4%, respectively, p=0.11 and p=0.08 vs. control).

Proteomics analysis comparing $HDL_{Healthy}$ and HDL_{CAD} using LC-ESI-MS/MS

We therefore used a mass spectrometric-based approach to identify changes in HDL-associated proteins in coronary disease. The spectral index was used for relative quantification of each protein of interest,[30, 31] as described in detail in the Supplementary Material.

Based on this approach, we identified 78 HDL-associated proteins with substantial differences in their quantitative abundance between $HDL_{Healthy}$ and HDL_{CAD} (Figure 3C, Supplementary Table 1). We further applied gene ontology analysis to identify proteins that are related to regulation of apoptosis (Supplementary Table 2). Based on these analyses two proteins, clusterin and apoC-III, were selected for further assessment of their potential relevance in mediating effects of HDL on endothelial apoptosis, given their differential abundance in HDL_{CAD} and their potential role in regulation of apoptotic processes.

Validation of altered clusterin levels in HDL_{CAD} as compared to $HDL_{Healthy}$ and its relevance for endothelial anti-apoptotic effects of HDL

Proteomics analysis indicated reduced clusterin levels in HDL_{CAD} relative to $HDL_{Healthy}$ (spectral index of -0.323) (Supplementary Table 1). An MS/MS fragmentation pattern of a proteotypic peptide of clusterin is shown in Figure 4A. To further validate this finding, we quantified clusterin in $HDL_{Healthy}$ and HDL_{CAD} isolated by two different methods, i.e. ultracentrifugation and gel filtration. Independent of the isolation method, substantially lower clusterin levels were detected in HDL_{sCAD} and HDL_{ACS} as compared to $HDL_{Healthy}$ (Figure 4B, Table 2).

To further investigate a potential functional role of clusterin in HDL for effects on endothelial apoptosis, we pre-incubated $HDL_{Healthy}$ with specific clusterin blocking antibodies (Santa Cruz Biotechnology, USA) and supplemented HDL_{CAD} with clusterin. Pre-incubation of $HDL_{Healthy}$ with clusterin-blocking antibodies (1:50 or 1:100 dilution), but not with an IgG isotype control, reduced endothelial anti-apoptotic effects of $HDL_{Healthy}$ in a dose-dependent manner as measured by annexin-V staining (Figure 4C). The presence of the clusterin-blocking antibody decreased the capacity of $HDL_{Healthy}$ to reduce endothelial cell apoptosis as measured by caspase-3 activity (Figure 4D) and TUNEL staining (apoptotic endothelial cells: 38.2±5.6% versus 23.1±6.2%, $p<0.05$). Similarly, the capacity of $HDL_{Healthy}$ to attenuate TNF-α-induced endothelial apoptosis was reduced in the presence of clusterin blocking antibody (Figure 4E). Vice versa, supplementation of HDL_{CAD} with purified clusterin increased its endothelial anti-apoptotic effects (Figure 4F). Notably, purified clusterin alone had no significant effect on endothelial apoptosis, indicating that binding of clusterin to the HDL particle was important for its full endothelial anti-apoptotic effects. To further investigate the role of HDL-associated clusterin for endothelial anti-apoptotic effects of HDL, we prepared reconstituted HDL with and without clusterin. Addition of clusterin to rHDL resulted in a more profound endothelial anti-apoptotic effect (Figure 4G), supporting a role of HDL-bound clusterin for endothelial anti-apoptotic properties of $HDL_{Healthy}$. Of note, corresponding amounts of HDL-bound clusterin after supplementation of reconstituted HDL or HDL_{CAD} were within a similar range as compared to clusterin levels observed in $HDL_{Healthy}$, further supporting the concept that reduced levels of HDL-bound clusterin in HDL_{CAD} impact on the capacity of HDL to limit endothelial apoptosis (Supplementary Figure 1A).

Increased apoC-III levels in HDL_{sCAD} and HDL_{ACS} and relevance for effects of HDL on endothelial apoptosis

Based on the spectral index of the proteomics analysis, levels of apoC-III were increased in HDL_{sCAD} and HDL_{ACS} as compared to $HDL_{Healthy}$ (spectral index of 0.197) (Supplementary Table 1). An MS/MS fragmentation pattern of a proteotypic peptide of apoC-III is shown in Figure 5A. To further validate this finding we quantified apoC-III in $HDL_{Healthy}$ and HDL_{CAD} isolated by either ultracentrifugation or gel filtration. HDL_{sCAD} and HDL_{ACS} were enriched with apoC-III as compared to $HDL_{Healthy}$ (Figure 5B, Table 2). Pre-incubation of HDL_{CAD} with a specific blocking antibody for apoC-III (1:50 dilution), but not with an isotype-control-antibody, improved its capacity to reduce endothelial apoptosis, both after serum withdrawal (Figures 5C-F) or TNF-α-exposure (Figures 5G-I). Similarly, the blocking antibody against apoC-III increased the endothelial anti-apoptotic capacity of HDL_{CAD} as measured by TUNEL staining (25.2±5.6% versus HDL_{CAD} alone 37.1±6.2%, $p<0.05$). Conversely, pre-incubation of $HDL_{Healthy}$ with purified apoC-III impaired its capacity to attenuate endothelial apoptosis (Figure 5J). The corresponding amounts of HDL-bound apoC-III after supplementation of $HDL_{Healthy}$ with apoC-III were in a similar range as compared to HDL-bound apoC-III levels observed in HDL_{CAD} (Supplementary Figure 1B).

$HDL_{Healthy}$ activates the endothelial anti-apoptotic Bcl-2 protein Bcl-xL via PI3K/Akt pathway, whereas HDL_{CAD} upregulates the endothelial pro-apoptotic Bcl-2 protein tBid via MAPK-p38 – role of HDL-associated clusterin and apoC-III.

Further assessment of signaling mechanisms involved in effects of HDL on endothelial apoptosis showed that the anti-apoptotic capacity of $HDL_{Healthy}$ was mediated via PI3K/Akt, since PI3K

inhibition by wortmannin or LY294002 prevented endothelial anti-apoptotic effects of $HDL_{Healthy}$, whereas these anti-apoptotic pathways were not activated by HDL_{CAD} (Figure 6A). $HDL_{Healthy}$ phosphorylated endothelial Akt at Ser473, which was not observed using HDL_{sCAD} or HDL_{ACS} (Supplementary Figure 2A).

Our further analysis of downstream targets of HDL on endothelial apoptotic pathways revealed a differential regulation of anti-apoptotic and pro-apoptotic proteins from the Bcl-2 family by $HDL_{Healthy}$ and HDL_{CAD}. $HDL_{Healthy}$ activated Bcl-xL, an anti-apoptotic Bcl-2 protein[32, 33] whereas HDL_{CAD} activated tBid, a pro-apoptotic Bcl-2 protein[34, 35] (Figures 6B and 6C). No significant differences were observed on the expression of other Bcl-2 proteins, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bak by $HDL_{Healthy}$ and HDL_{CAD} (*data not shown*). Wortmannin or LY294002 inhibited effects of $HDL_{Healthy}$ on Bcl-xL expression (Figure 6D). Notably, pre-incubation of $HDL_{Healthy}$ with clusterin blocking antibody reduced HDL-dependent phosphorylation of Akt at Ser473 (Figure 6E) and impaired upregulation of Bcl-xL expression (Figure 6F). These findings indicate that clusterin contributes to effects of $HDL_{Healthy}$ on stimulation of PI3K/Akt which in turn modulates endothelial expression of the anti-apoptotic Bcl-xL.

Furthermore, assessment of the role of mitogen-activated protein kinase (MAPK) demonstrated that HDL_{CAD} increased endothelial phosphorylation of p38-MAPK, whereas $HDL_{Healthy}$ had no effect (Figure 7A). Inhibition of HDL_{CAD} induced p38-MAPK-activation using the inhibitor SB203580 blocked the increase in endothelial expression of tBid (Figure 7B). Pre-incubation of HDL_{CAD} with the specific blocking antibody against apoC-III inhibited the increased

phosphorylation of p38 MAPK (Figure 7C) and the increased expression of tBid (Figure 7D). These observations suggest that HDL-associated apoC-III stimulates pro-apoptotic signaling by phosphorylation of p38-MAPK and upregulation of endothelial expression of pro-apoptotic tBid.

Assessment of clusterin and apoC-III content in other lipoprotein fractions

The content of clusterin in serum or in the LDL/VLDL fraction was not reduced in patients with CAD as compared to healthy subjects (Table 2), whereas clusterin levels were substantially lower in HDL_{CAD} as compared to $HDL_{Healthy}$ isolated by both ultracentrifugation or gel filtration methods, compatible with the concept of a reduced binding of clusterin to HDL_{CAD} (Table 2).

The apoC-III content was mainly increased in HDL_{CAD}, but was also to some extent elevated in serum and the LDL/VLDL fraction of patients with CAD as compared to healthy subjects using both, ultracentrifugation or gel filtration lipoprotein isolation protocols (Table 2). These observations suggest a systemic increase of apoC-III levels in patients with CAD.

Role of SR-BI for endothelial anti-apoptotic effects of HDL

Pre-incubation of endothelial cells with blocking antibody against SR-BI inhibited endothelial anti-apoptotic effects of $HDL_{Healthy}$ (Supplementary Figures 3A-B). Further studies on clusterin or apoCIII and SR-BI-dependent endothelial binding of HDL are described in the supplementary file. The SR-BI blocking antibody also reduced the anti-apoptotic capacity of rHDL/Clu (Supplementary Figure 3A). These observations suggest that the capacity of HDL-associated clusterin to reduce endothelial apoptosis is mediated, at least in part, via SR-BI.

DISCUSSION

In the present study we have for the first time compared the effects of HDL from healthy subjects and from patients with CAD on endothelial cell anti- and pro-apoptotic signaling pathways. Importantly, while $HDL_{Healthy}$ substantially reduced endothelial cell apoptosis *in vitro* and *in vivo*, no such effects were observed for HDL from patients with sCAD or ACS.

Of note, our studies revealed differential effects of $HDL_{Healthy}$ and HDL_{CAD} on endothelial anti- and pro-apoptotic signaling pathways, in particular on members of the Bcl-2 family of proteins, that are critical regulators of apoptosis. $HDL_{Healthy}$, but not HDL_{CAD}, activated the endothelial anti-apoptotic Bcl-xL pathway. In contrast, HDL_{CAD} activated endothelial tBid, a pro-apoptotic Bcl-2 protein. Our studies further suggest that differences in the proteome of HDL_{CAD}, in particular reduced HDL-associated clusterin and increased HDL-associated apoC-III, play an important role for altered activation of endothelial anti- and pro-apoptotic signaling pathways (Figure 8).

Endothelial dysfunction and injury are thought to play an important role in initiation and progression of atherosclerotic CAD.[13-15] Experimental studies have shown that atherosclerotic lesion-prone vascular regions such as bifurcations are characterized by high endothelial cell turn-over,[16] likely indicating an increased rate of endothelial cell apoptosis.[22] Furthermore, coronary atherosclerotic plaque erosion with loss of an intact endothelial cell monolayer is frequently observed in patients with an acute coronary syndrome,[18, 19] and areas of endothelial denudation may promote superficial thrombosis and progression of coronary atherosclerosis.[17, 36, 37] The

capacity of HDL to inhibit endothelial cell apoptosis has therefore been suggested as an important potential anti-atherogenic property of HDL.[23-26]

In the present study delipidated $HDL_{Healthy}$ exerted a more profound endothelial anti-apoptotic effect as compared to purified or recombinant apoA1 and reconstituted HDL, compatible with the concept that the HDL proteome is important for the regulation of endothelial anti-apoptotic pathways by HDL. Our HDL proteomics and gene ontology analysis suggested that changes in HDL-bound clusterin and apoC-III in HDL_{CAD} could be relevant for altered effects on apoptosis. Subsequent validation experiments using HDL obtained by different preparative methods provided evidence that a reduced HDL-associated clusterin and an increased HDL-associated apoC-III content contribute importantly to altered effects of HDL_{CAD} on endothelial apoptosis. A previous study by Vaisar et al.[31] on shotgun proteomics analysis of HDL observed a trend for a lower clusterin and a higher apoC-III content in HDL from patients with CAD according to the peptide index analysis, however, no validation experiments were performed. The present study demonstrates for the first time that proteome remodeling in HDL_{CAD} has direct implications on functional properties, i.e. vascular effects of HDL. These findings point to a novel mechanism leading to altered vascular effects of HDL in patients with coronary disease, that at present is of particular interest, given the disappointing results of several clinical trials examining therapeutic strategies of HDL-cholesterol raising in these patients.

Clusterin overexpression via adenovirus transfection has been suggested to render endothelial cells more resistant against TNF-α-induced apoptosis.[38] In the present study, however, we have examined the effects of HDL-bound clusterin on endothelial apoptosis using extracellular

administration which represents a different setting as compared to overexpression in cultured cells. Notably, addition of clusterin alone did not result in an endothelial anti-apoptotic effect, that was only observed when clusterin was bound to HDL. Clusterin has been suggested to associate with apoA-I[39] and paraoxonase-1.[40] A recent study by Hoofnagle et al.[41] observed that HDL-clusterin levels are reduced in insulin resistant men with a high body-mass-index. It was therefore speculated that a reduced HDL-clusterin-content may have functional implications for vascular effects of HDL. Of note, a potential anti-atherogenic role of clusterin has been supported by studies examining administration of a clusterin-peptide fragment to apoE-deficient mice and monkeys.[42] In a study of 6 patients with coronary disease it has been observed that combined statin/niacin therapy may promote higher HDL-clusterin-levels in patients with coronary disease.[43] Our present findings provide novel evidence that reduced HDL-clusterin-levels are functionally important with respect to the impaired capacity of HDL_{CAD} to exert endothelial anti-apoptotic effects.

Serum clusterin levels have been reported to be increased in patients with developing coronary heart disease, or myocardial infarction, or type II diabetes[44] and also in response to endotoxin and cytokines[45]. In the present study there was a trend towards increased clusterin-serum-levels. These observations further suggest that changes in the clusterin-content of HDL_{CAD} are specific for HDL, and that the underlying cause is likely a reduced association of clusterin with HDL in patients with CAD rather than systemically reduced clusterin levels.

We observed that HDL-associated clusterin activates via PI3K/Akt the endothelial anti-apoptotic protein Bcl-xL, Bcl-xL belongs to the family of Bcl-2 proteins,[32] has been proposed to be

regulated by Akt in tumor cells,[46] and was reported as an important anti-apoptotic protein in endothelial and other cell types.[33] The capacity of $HDL_{Healthy}$ to reduce endothelial apoptosis was not abolished when eNOS was inhibited, although the activation of PI3K/Akt was required for the anti-apoptotic activity of HDL. In line with this observation, a study by Suc et al. has suggested that the protective effect of HDL on endothelial cell survival was not dependent on HDL-associated paraoxonase activity,[25] that we have observed to be critical for the capacity of HDL to stimulate endothelial NO production.[10]

In addition, our study demonstrates that increased levels of apoC-III associated with HDL_{CAD} were responsible for activation of the pro-apoptotic p38-MAPK-signaling pathway in endothelial cells followed by increased expression of the pro-apoptotic protein tBid, a member of Bcl-2 family of proteins.[34, 47] Increased apoC-III levels have been associated with hypertriglyceridemia, metabolic syndrome and diabetes.[48-50] ApoC-III inhibits the clearance of triglyceride-rich lipoproteins.[51] A previous study has shown that LDL containing apoC-III was independently associated with an increased risk of CAD.[52] Furthermore, a recent case-control study has suggested that HDL containing apoC-III is associated with a higher risk of future CHD.[53] Transfers of apoC-III between lipoprotein particles have been previously described.[54] However, in the present study the apoC-III content was not only increased in the HDL fraction but also in the serum and the LDL/VLDL fraction of patients with CAD, suggesting an increased synthesis or a reduced clearance of apoC-III in patients with CAD.

Of note, our findings do not exclude that HDL-associated lipids may also promote endothelial anti-apoptotic effects of HDL. In particular, HDL-associated lysosphingolipids have been

suggested to exert endothelial anti-apoptotic effects,[23] although this has not been observed when sphingosine-1-phosphate (S1P) was added to reconstituted HDL.[26] Interestingly, however, a recent study has examined the S1P content of HDL in patients with coronary disease and an acute coronary syndrome.[55] Notably, in patients within the first hours of an acute coronary syndrome an increased S1P content of HDL was observed, suggesting that it would be unlikely that an altered S1P content could explain the loss of endothelial anti-apoptotic effects of HDL in these patients.[55]

A recent study by Khera et al.[12] supports the notion that HDL function may be more relevant as compared to HDL-cholesterol serum levels alone for development of coronary disease. Khera et al. analyzed the cholesterol efflux capacity of apoB-depleted serum, that was lower in cases with coronary disease as compared to controls, independently of HDL-cholesterol levels. The present study describes for the first time, that effects of HDL on endothelial cell apoptosis are markedly different between $HDL_{Healthy}$ and $HDL_{CAD,}$ that may limit the capacity of HDL to counteract clinical complications of atherosclerosis, since an impaired endothelial integrity has been suggested as an important mechanism promoting clinical complications of coronary disease.

Study limitations. A potential limitation of the present study is the use of cardiovascular drugs in patients with CAD, but not in healthy subjects, that may impact on functional properties and composition of HDL. Previous studies have suggested that statin therapy may reduce apoC-III levels in plasma, HDL or apoB-containing lipoproteins.[56, 57] In the present study most patients were on statin therapy, however, apoC-III levels in LDL/VLDL and HDL were still higher as compared to healthy subjects. While we cannot exclude that medical treatment in addition to the

underlying coronary disease may have impacted on altered HDL composition and function in patients with CAD, the present data are consistent with the notion that despite current medical therapy the function and composition of HDL remain abnormal as compared to healthy subjects. Another potential limitation of our study is the use of chloroform/methanol method for delipidation of HDL where we cannot exclude extraction of hydrophobic proteins from HDL. However, since delipidated HDL still exerted a similar effect on endothelial apoptosis as compared to $HDL_{Healthy}$ before lipid extraction (Figure 3B), the anti-apoptotic protein components were likely largely present in the delipidated HDL protein fraction.

In summary, our findings provide novel evidence that remodeling of the HDL proteome in patients with coronary disease has important functional implications with respect to effects of HDL on endothelial cell survival. In particular, we have observed that a reduced clusterin and increased apoC-III content in HDL_{CAD} lead to an impaired effect of HDL on endothelial anti-apoptotic pathways and an activation of pro-apoptotic-signaling in endothelial cells. These findings provide novel insights into mechanisms underlying altered vascular effects of HDL in patients with coronary disease.

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DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1 Effects of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} on endothelial apoptosis *in vitro*. (A) The impact of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} (50µg/ml based on protein measurement) on endothelial apoptosis was determined by annexin-V staining using FACS analysis after 16 hours of serum deprivation (n=20 per group) (B) Representative examples of flow cytometry analyses of annexin-V staining. Dark grey solid line: control cells for autofluoresence, Light grey dotted line: serum withdrawal, Red line: HDL-treated cells with serum withdrawal. (C) Endothelial apoptosis in the absence or presence of HDL (50µg/ml) was determined for the indicated time intervals following serum/growth factor deprivation. The dotted line represents buffer-treated cells with serum withdrawal. (D) Caspase-3 activity was measured from the lysate of endothelial cells treated with or without HDL (50µg/ml; n=20 per group). (E,F) Fluorescence microscopy of apoptotic endothelial cells stained with annexin-V-FITC. Number of annexin-V+ cells was counted per high power field (average values were taken from 5 hp-fields). (G) Effect of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} (50µg/ml) on endothelial cell apoptosis induced by TNF-α as measured by annexin-V staining using FACS analysis.

Figure 2 Effects of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} on endothelial apoptosis *in vivo*. (A) Administration of $HDL_{Healthy}$, but not HDL_{CAD} (14mg HDL protein/kg body weight) to ApoE-/- mice via tail-vein injection reduced endothelial apoptosis as detected in the aorta after 24 hours (n=8-9 per group) as measured by co-staining of annexin-V+ and CD31+ using FACS analysis. (B) Representative flow cytometric analyses using anti-CD31-PE / annexin-V-APC co-staining of cells harvested from mouse aortas. (C) Immunofluorescence staining showed reduced active

caspase-3 staining of endothelial cells of mouse aortic sections after treatment with $HDL_{Healthy}$, but not with $HDL_{CAD.}$ (D,E) TUNEL-FITC staining of mouse aortic sections co-stained with CD31-Texas-red. Number of TUNEL+ cells / Number of CD31+ cells was counted per high power field and the average was taken from 5 fields.

Figure 3 Evaluation of the role of endothelial NO synthase and the effects of different HDL components ($HDL_{Healthy}$) on endothelial apoptosis (A) $HDL_{Healthy}$ reduced endothelial apoptosis after inhibition of eNOS-mediated endothelial NO production by siRNA knockdown of *eNOS* or treatment with L-NAME (1 mM) (n=6-8 per group). Scr: scrambled. (B) Effects of $HDL_{Healthy}$ (50µg/ml), delipidated HDL (50µg/ml), apo A-1 (50µg/ml) or reconstituted HDL (50µg/ml) on endothelial apoptosis as measured by FACS analysis with annexin-V staining (n=6 per group). Reconstituted HDL was prepared with the sodium cholate dialysis method using an apoA-I/POPC/cholesterol molar ratio of 1:80:10. (B) LC-ESI-MS/MS analysis of $HDL_{Healthy}$ and HDL_{CAD} (n=6 per group). Proteins identified were quantified using spectral index and data are presented as proteins reduced or enriched in HDL_{CAD}.

Figure 4 Role of HDL-associated clusterin for effects of $HDL_{Healthy}$ on endothelial anti-apoptotic pathways. (A) MS/MS spectrum of a proteotypic peptide of clusterin. (B) Concentration of clusterin associated with $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} **as** quantified using ELISA. (C) $HDL_{Healthy}$ (50µg/ml) was pre-incubated with specific blocking antibody against clusterin (2-4µg/ml) and the effects of HDL on endothelial cell apoptosis induced by serum withdrawal were analyzed using annexin-V staining by FACS analysis, and (D) caspase-3 activity assay. (E) Effect of the clusterin blocking antibody on the anti-apoptotic capacity of

$HDL_{Healthy}$ (50μg/ml) as examined in TNF-α stimulated endothelial cells. (F) Effects of HDL_{CAD} (50μg/ml) on endothelial cell apoptosis in the presence of increasing concentrations of purified human clusterin (2.5-10μg/ml) were determined. (E) Effects of reconstituted HDL (50μg/ml) in the absence or presence of clusterin on endothelial cell apoptosis were analyzed.

Figure 5 Role of HDL-associated apoC-III for impaired endothelial anti-apoptotic effects of HDL in patients with CAD. (A) MS/MS spectrum of a proteotypic peptide of apoC-III. (B) The amount of apoC-III in $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} was quantified with ELISA. (C,D,E) HDL_{CAD} (50μg/ml) was pre-incubated with specific blocking antibody against apoC-III (20μg/ml or 1:50 dilution) or isotype control and the effects of HDL on serum withdrawal induced-endothelial apoptosis were analyzed with annexin-V staining using FACS analysis and (F) caspase-3 activity measurement. (G,H,I) Effects of HDL_{CAD} pre-incubated with a specific blocking antibody against apoC-III or isotype control on TNF-α-induced-endothelial apoptosis were analyzed with annexin-V staining using FACS analysis. (J) Effects of $HDL_{Healthy}$ (50μg/ml) on endothelial cell apoptosis in the presence of purified human apoC-III (5μg/ml).

Figure 6 Activation of the endothelial anti-apoptotic signaling pathways by $HDL_{Healthy}$. (A) Pre-treatment of endothelial cells with PI3K inhibitors, wortmannin (100nM) or LY294002 (50μM) reversed the anti-apoptotic effects of $HDL_{Healthy}$ (50μg/ml). (B) Effects of $HDL_{Healthy}$, HDL_{sCAD} or HDL_{ACS} (50μg/ml) on the expression of endothelial Bcl-xL expression and (C) endothelial tBid expression were assessed by western blot analysis (n=6 per group). (D) Effects of $HDL_{Healthy}$ (50μg/ml) on Bcl-xL expression in HAECs pre-treated with PI3K inhibitor as analyzed by western blot analysis. (E) Effects of $HDL_{Healthy}$ pre-incubated with specific blocking

antibody against clusterin (4µg/ml) on the phosphorylation of Akt at Ser473, (F) Bcl-xL expression (n=6 per group), as detected by western blot analysis.

Figure 7 Stimulation of the endothelial pro-apoptotic signaling pathways by HDL_{CAD}. (A) Effects of $HDL_{Healthy}$ and HDL_{CAD} on phosphorylation of MAPK p38 were analyzed by western blot analysis (n=8-10 per group). (B) Effects of HDL_{CAD} on endothelial tBid expression pre-incubated with SB203580 (10µM). (C) Effects of HDL_{CAD} pre-incubated with specific blocking antibody against apoC-III on the phosphorylation of MAPK p38, (D) tBid expression (n=6-8 per group), as detected by western blot analysis.

Figure 8 Summary of major findings of the study. HDL proteome remodeling in CAD leads to altered effects on endothelial anti-apoptotic and pro-apoptotic pathways. $HDL_{Healthy}$ carries higher amounts of clusterin, that upon induction of apoptosis promotes activation of endothelial PI3K/Akt leading to increased expression of anti-apoptotic Bcl-xL. In CAD, the level of HDL-associated clusterin is reduced, whereas HDL-associated apoC-III content is increased. HDL-associated apoC-III activates MAPK signaling via phosphorylation of p38 leading to increased activation of pro-apoptotic tBid. Our findings therefore suggest that HDL proteome alterations in CAD have implications for the function of HDL with respect to its effects on endothelial integrity and survival.